Effects of sucrose and omega-3 dietary modification on lipid peroxidation in the liver of mouse models of breast cancer and chemotherapy

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Abstract

Doxorubicin, a widely used chemotherapeutic agent, generates free radicals which may increase lipid peroxidation and oxidative stress. Research suggests that sucrose increases oxidative stress; conversely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) limit oxidative stress and lipid peroxidation in the brain of mice treated with doxorubicin. It is unknown how the combination of sucrose and omega-3 fatty acids impacts lipid peroxidation in metabolically important tissues such as the liver in the presence of chemotherapy or mammary tumors. Livers from mouse models of breast cancer (Tumor model: n=32; 8/diet) and chemotherapy (Chemo model: n=40;10/diet) were analyzed for 4-hydroxynonenal (4-HNE), a lipid peroxidation marker, using an enzyme-linked immunoabsorbent assay. In both experiments, 8-9 week old female C57BL/6 mice were ovariectomized and randomized to diets one week later. Tumor model mice were fed low sucrose diets, with either 0% or 2% kcal from EPA+DHA. Two weeks later, mice were injected with metastatic mammary tumor cells or control; after 21 days, tissues were collected. Chemo model mice were fed low sucrose, 0% or 2% kcal EPA+DHA diets, or high sucrose, 0% or 2% kcal EPA+DHA diets. Mice were injected with doxorubicin based chemotherapy or saline two and four weeks later; tissues were collected 11 days after second injection. Mean liver 4-HNE in tumor mice (0.0649ug HNE-BSA/ugProtein; SEM 0.0066) vs. control (0.0613ug HNE-BSA/ugProtein; SEM 0.0066) was not significantly different (p=0.71) and did not differ by diet (p=0.54). Similarly, mean liver 4-HNE in chemo mice (0.0817ug HNE-BSA/ugProtein; SEM 0.0114) vs. control (0.0877ug HNE-BSA/ugProtein; SEM 0.0117) was not significantly different (p=0.72) and did not differ by diet (p=0.42). In conclusion, we found no differences in liver lipid peroxidation in chemotherapy-treated or tumor mice vs. controls when fed varying levels of sucrose and EPA+DHA.

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Introduction

In the United States of America (US), breast cancer is the second leading cause of death in women[6]. Doxorubicin and cyclophosphamide are widely used as antitumor drugs for treatment of cancer, in particular breast cancer[10,15]. But, an exposure to doxorubicin through chemotherapy could mediate generation of free radicals in the brain tissues as it promotes lipid peroxidation which changes the antioxidant defense system and may lead to neuropsychological changes in mice model[15]. Doxorubicin's anticancer action is believed to be caused by free radical generation through redox reaction which causes DNA damage, however the mechanism of free radical formation by doxorubicin remains unknown[9]. Also, the usage of cyclophosphamide kills immune cells in the body through selective suppression of regulatory T cells and that affects the therapeutic efficacy of chemotherapy[10]. In a recent review regarding rodents' brains, it was mentioned that the addition of omega-3 fatty acids especially EPA and DHA in the diet has been known for reducing oxidative stress in the brains of male rats[12]. In contrast, high levels of dietary sucrose were associated with increasing lipid peroxidation in brain of mouse models as well as counteracting the anti-inflammatory effect of the omega-3 fatty acids[1,8]. 4-hydroxynonenal (4-HNE) is one of the end-products and biomarkers of lipid peroxidation and readily generated in brain and liver of mice in response to presence of toxicants like doxorubicin[16]. However, lipid peroxidation after feeding a range of sucrose and omega-3 fatty acids at levels typically consumed by women undergoing chemotherapy for breast cancer have yet to be studied. Additionally, it is still not known how the combination of sucrose and omega-3 fatty acids impact lipid peroxidation in the liver when chemotherapy is administered. It is important to look at liver's response to the chemotherapy treatment and varying levels of sucrose and omega-3 fatty acids because it is a major organ responsible for detoxification of drugs as well as synthetic function of many biochemical

pathways[13]. This study was aimed to investigate the effects of sucrose and omega-3 dietary modification on lipid peroxidation in the liver of mouse models of breast cancer and chemotherapy.

Literature review

In this literature review, relevant studies are grouped into three categories: effects of mammary tumor and omega-3 fatty acids on lipid peroxidation in the liver; effects of chemotherapy and omega-3 fatty acids on lipid peroxidation in the liver; effects of omega-3 fatty acids and sucrose on lipid peroxidation in the liver.

Mammary tumors, omega-3 fatty acids and lipid peroxidation in the liver

There have been very few studies that were designed to examine the relationship between mammary tumor, omega-3 fatty acids and lipid peroxidation. Also, they did not measure lipid peroxidation via 4-HNE but instead with phospholipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) which are also end-products of lipid peroxidation as well. In a study by Kikugawa et al., (2003) the effect of omega-3 fatty acid supplementation on oxidative stress-induced DNA damage of rat hepatocytes was studied using 32, four weeks old, male Wistar rats which were fed with 50g/kg dried diets consisting of either fish oil rich in EPA and DHA or safflower oil rich in omega-6 fatty acids and as well as equal amounts of vitamin E in 59g/kg of dried diet for 6 weeks[5]. The hepatocytes were isolated and exposed with *in vitro* hydrogen peroxide to induce oxidative stress and the lipid peroxidation level was measured with phospholipid hydroperoxides and thiobarbituric acid-reactive substances[5]. It was concluded that the level of lipid-peroxidation of hepatocytes increased slightly more in the omega-3 fatty acids group compared to the omega-6 fatty acids group[5]. Based on the above research, omega-3 fatty acids specifically EPA and DHA may produce higher levels of lipid peroxidation compared to omega-6 fatty acids[5].

Another study looked at the effect of different ratios of dietary corn oil and fish oil and the influence of antioxidant addition on the growth and lipid peroxidation on MDA- MB231 mammary

tumor in athymic nude mice[4]. 164, four to five weeks old, female athymic nude mice were used in the study and were fed with varying levels of corn oil and fish oil at different ratios; one of the diet had antioxidants added into it[4]. The diets were fed for 6 to 8 weeks starting 7 to 10 days after the administration of human breast carcinoma MDA-MB231[4]. TBARS were used to detect the level of lipid peroxidation and it was reported that the TBARS level in the human breast carcinomas increased in mice fed with fish oil diets without antioxidants[4]. That accumulation of TBAR levels reportedly helped to suppress tumor growth[4]. There was a direct relationship between the level of TBARS and level of fish oil fed[4]. The study showed positive effect of lipid peroxidation in mammary tumor as the growth of the tumor was suppressed.

EPA and DHA have also been extensively studied for their effects on breast cancer[7]. Many studies were done in xenograft and transgenic mouse models and generally showed benefits of EPA and DHA[7]. Liu and Ma's (2014) review mentioned breast cancer studies in chemically-induced mice models supported omega-3 fatty acids' anti-cancer effects[7]. One of the studies mentioned in the review by Olivo and Hilakivi-Clarke (2005), compared the effects of varying levels of EPA and DHA and omega-6 fatty acids respectively on mammary tumorigenesis in rats[11]. The rats fed with a low omega-3 fatty acids diet showed elevated lipid peroxidation but lower incidence of mammary tumors when compared to mice-fed omega-6 fatty acids[11]. The review showed that EPA and DHA have mixed effects on mammary tumors.

Chemotherapy, omega-3 fatty acids and lipid peroxidation in the liver

A study by Bhattacharya et al., (2013) looked at effect of dietary omega-3 and cyclophosphamide, a common chemotherapeutic agent, on lipid peroxidation in livers of autoimmune-prone NZB/W female mice[2]. Female NZB/W mice (n=40, two months old) were fed with either *ad libitum* or food restricted diets containing 5% corn oil or 5% fish oil supplemented

with equal levels of antioxidants[2]. Cyclophosphamide was used instead of doxorubicin but it is also an agent used to treat cancer[13]. The cyclophosphamide was injected every 10 days for 10 months but the diet continued up until 12 months before being sacrificed[2]. The liver was analyzed for lipid peroxidation via TBARS and it was reported that omega-3 fatty acids with *ad libitum* dietary group exhibited more susceptibility to lipid peroxidation especially with the presence of cyclophosphamide than that of omega-6 fatty acids[2].

Omega-3 fatty acids and sucrose on lipid peroxidation in the liver

Ma et al., (2011) looked at sucrose-based high fat diet and sucrose's effect on the anti-inflammatory effect of fish oil in adipose tissue and obesity development in C57BL/6J male mice for 9 weeks[8]. The type of diets fed consisted of corn oil or fish oil supplemented with either protein or sucrose or a conventional low fat diet *ad libitum*[8]. It was reported that sucrose counteracted the anti-inflammatory effects of fish oil in adipose tissue[8]. However, sucrose did not reduce the ability of fish oil to prevent diet-induced accumulation of fat in the liver, which could decrease the amount of lipid peroxidation in the liver[8]. This study showed that omega-3 fatty acids may and may not be beneficial at limiting lipid peroxidation in the liver when sucrose is present.

The aim of this thesis project was to determine differences in lipid peroxidation, detected via 4-HNE, induced by diets of varying levels of sucrose and omega-3 fatty acids in ovariectomized, female mice after exposing them to mammary tumor or chemotherapy injections. This thesis focused on the liver as it is understudied in both mammary tumor and chemotherapy mice models. It is important to understand the liver and its function after exposure to mammary tumor and chemotherapy as it is the site responsible for metabolism of many commonly used anticancer agents[4].

Materials and Methods

Study design

The analysis was performed on liver tissues from two different experiments which involved mouse models of mammary tumor and chemotherapy. Eight to nine week old, ovariectomized female (C57BL/6) mice were used for both mouse models. Figure 1 shows the timeline of the mammary tumor mouse model prior to laboratory analysis and Figure 2 shows the timeline of the chemotherapy mouse model prior to laboratory analysis.

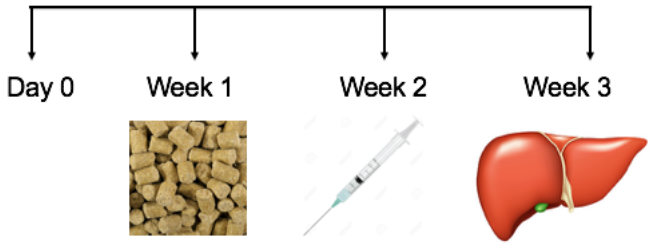


Figure 1: Timeline of the mammary tumor mouse model with the mice being ovariectomized on Day 0, fed with the different diets through pellet starting on Week 1 and continuing through end of study, tumor injection (E0771 metastatic mammary tumor cell line) on Week 2, and tissue collection, flash frozen with liquid nitrogen, and stored in freezer (-80ºC) on Week 3

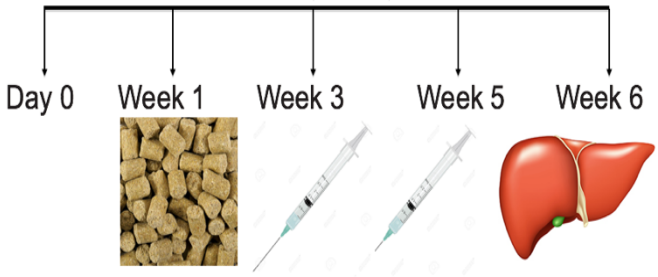


Figure 2: Timeline of the chemotherapy mouse model with the mice being ovariectomized on Day 0, fed with the different diets through pellet starting on Week 1 and continuing through end of study, chemotherapy (50% Human Equivalent dose of 9mg/kg Doxorubicin and 90mg/kg

Cyclophosphamide) or saline injection on Week 3 and Week 5, and tissue collection, flash frozen with liquid nitrogen, and stored in freezer (-80ºC) on Week 6

Mammary tumor mouse model

Treatment

The mice were injected with Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher, Waltham, MA), which was used as the control or with tumor cells (CH3 BioSystems, Amherst, NY). The tumor injected mice had E0771 metastatic mammary tumor cell line using intraperitoneal injections of 10^6 cells bilaterally.

Diet

An ingredient list consisting of the different mouse diets could be found in Appendix B. The total amount of omega-3 fatty acids used in the diets with 2% EPA+DHA was 23% (Appendix C). The ratio of omega-3 and omega-6 in the diets with 0% EPA +DHA was 1:7 while the ratio of omega-3 and omega-6 in the diets with 2% EPA+DHA was 1: 1.3 (Appendix C). In the mammary tumor mouse model, two types of diet: low sucrose, 0% EPA + DHA and low sucrose, 2% EPA + DHA were used to feed the mice in the form of pellet. The compositions of the different diets are tabulated in Table 1. A total of 58 mice were used for both types of treatment but for 4-HNE analysis, 32 mice were randomly chosen with 8 per diet group.

Table 1: Types of diet and their composition used in the mammary tumor mouse model

Diet	Sucrose (% kcal)	Omega-3 fatty acids (% kcal)
Low sucrose, 0% EPA+DHA	9 % kcal	0 % kcal
Low sucrose, 2% EPA+DHA	9 % kcal	2 % kcal (EPA:DHA ratio = 1.5:1)

Chemotherapy mouse model

Treatment

The mice were injected with saline, which was used as the vehicle or with doxorubicin and cyclophosphamide via tail vein injections two weeks and four weeks after starting diets. The chemotherapy treated mice were injected with 50% Human Equivalent dose (9mg/kg Doxorubicin and 90mg/kg Cyclophosphamide) (Pfizer, New York City, New York; Baxter International, Deerfield, IL).

Diet

An ingredient list consisting of the different mouse diets could be found in Appendix B. The total amount of omega-3 fatty acids used in the diets with 2% EPA+DHA was 23% (Appendix C). The ratio of omega-3 and omega-6 in the diets with 0% EPA +DHA was 1:7 while the ratio of omega-3 and omega-6 in the diets with 2% EPA+DHA was 1: 1.3 (Appendix C). In the chemotherapy mouse model, four types of diet which were used: low sucrose, 2% EPA+DHA diet, low sucrose, 0% EPA+DHA diet, high sucrose, 2% EPA+DHA diet, and high sucrose, 0% EPA+DHA diet. The compositions of different diets are tabulated in Table 2. A total of 102 mice were used in the experiment but for 4-HNE analysis, liver samples from 40 mice were randomly chosen with 10 samples per diet group.

Table 2: Types of diet and their composition used in the chemotherapy mouse model

Diet	Sucrose (% kcal)	Omega-3 fatty acids (% kcal)
Low sucrose, 2% EPA+DHA	9% kcal	2% kcal (EPA: DHA ratio = 1.5:1)
Low sucrose, 0% EPA+DHA	9% kcal	0% kcal

High sucrose, 2% EPA+DHA	47% kcal	2% kcal (EPA: DHA ratio = 1.5:1)
High sucrose, 0% EPA+DHA	45% kcal	0% kcal

Laboratory Analysis

Protein analysis

Protein lysate from mouse liver samples were prepared by homogenizing with 1 X PBS with protease inhibitor cocktail, then centrifuged at 16100 rcf for 15 minutes at 4°C and collect supernatant. Next, Bicinchoninic Acid (BCA) Protein Assay (Fisher Scientific, Waltham, MA) was conducted to measure the protein in liver lysate. Five microliter of protein lysates were added to 96-wells plate with 20 uL of lysis buffer (i.e., 1 X PBS with protease inhibitor cocktail) to dilute the sample. Then 200 uL working reagent (i.e., 50:1 of Reagent A and Reagent B) to each well. After 96-wells plate was incubated at 37 C for 30 minutes, absorbance was read with Synergy™ H1 hybrid microplate reader (Biotek, Winooski, VT) at 562 nm. The protein concentration was quantified regarding to a known concentration standard curve. The level of protein in samples was later used to normalize 4-HNE levels.

HNE Protein Adduct

The enzyme-linked immunoabsorbent assay (ELISA) kit (Cell Biolabs Inc., San Diego, CA) (Appendix A) was then used to analyze 4-HNE levels. The protein adducts were quantified through the comparison of the protein adducts' absorbance with a known standard curve. Firstly the HNE conjugate coated 96-wells plate was prepared by overnight incubation with 100 uL of 1X Conjugated Diluent containing 10 ug/mL HNE Conjugate at 4ºC on plate shaker.

The next day, the conjugate coated plate was washed twice with 200 uL of 1X PBS before use. Then 200 uL of assay diluent was pipetted into the 96-wells plate and incubated on plate shaker at room temperature. The HNE-BSA standard was prepared according to the kit manual while waiting for the plate incubation. After incubation, 50 uL of standard and samples were pipetted into the wells and then incubated for 10 minutes at room temperature on plate shaker.

Next, 50 uL of diluted Anti-HNE antibody was pipetted into each well and left for incubation for an hour at room temperature on plate shaker. The primary antibody was used to detect specific HNE protein in samples. When the incubation period was done, the plate was washed 3 times with 250 uL of 1X wash buffer. The washing step was done to remove unbound reagents and help to increase the sensitivity of the ELISA kit. One hundred microliter of diluted Secondary Antibody-HRP Conjugate was added to the wells and incubate for hour room temperature on plate shaker. The washing step was repeated and 100 uL of substrate solution was added. The incubation period was 20 minutes at room temperature on plate shaker. The stop solution was added at 100 uL into each well after the incubation period. The HNE protein adduct absorbance was read with the spectrophotometer at 450 nm. The level of HNE protein adducts in samples is calculated by comparison with a known HNE-BSA standard curve.

Statistical Analysis

All statistical analysis was performed via JMP (SAS Institute Inc, Cary, NC). The student's t-test was used to determine significant differences between the mean liver 4-HNE levels of different types of diet and the mean liver 4-HNE levels of the types of treatment in the two models. While the Tukey Kramer's test assessed for interactions between for different treatments and different diets used in each mouse model. Results were considered statistically significant at a level of $p < 0.05$.

Results

Mammary tumor mouse model

Mean and standard error of mean are presented in Table 3. There were no statistically significant differences in mean 4-HNE by tumor or control group (Figure 3). Using two-way ANOVA analysis, we explored trends for differences by diet groups (Figure 4) and within diet and treatment groups (Figure 5).

Table 3: Descriptive analysis of liver 4-HNE in mammary tumor mouse model

Condition	**Variable**		**Mean of 4-HNE ± standard error of mean (SEM) (ug HNE-BSA/ugProtein)**
Comparison of all treatments (Figure 3)	Control		0.0649 ± 0.0066
	Tumor		0.0613 ± 0.0066
Comparison of single diet to all treatments (Figure 4)	2% kcal EPA+DHA diet	Control	0.0564 ± 0.0079
		Tumor	0.0649 ± 0.0079
	0% kcal EPA+DHA diet	Control	0.0656 ± 0.0108
		Tumor	0.0648 ± 0.0108
Comparison of all diets (Figure 5)	2% kcal EPA + DHA diet		0.0607 ± 0.0069
	0% kcal EPA + DHA diet		0.0652 ± 0.0064

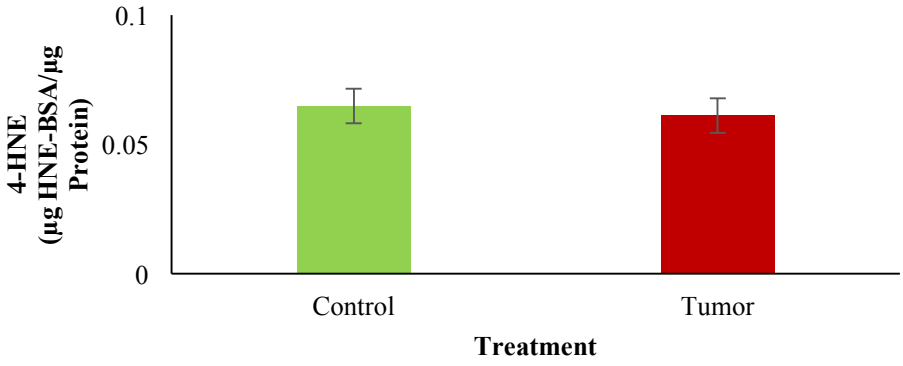
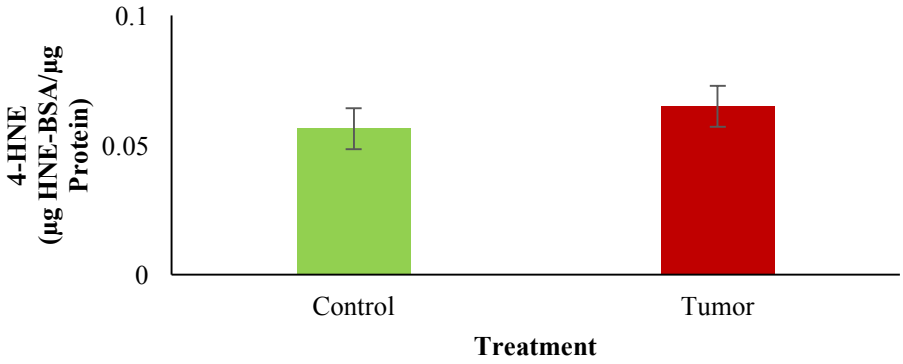


Figure 3: Comparison of liver 4-HNE in tumor vs control mice, without respect to diet group. p value = 0.7091(2 sample t-test)

2% EPA+DHA Diet



0% EPA+DHA Diet

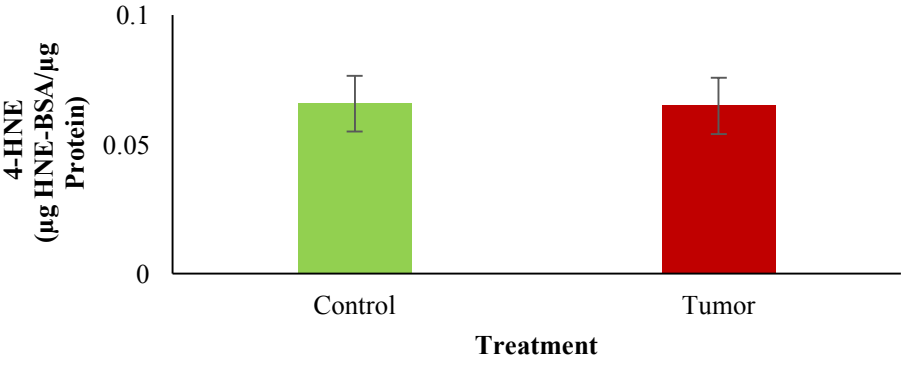


Figure 4: Comparison of liver 4-HNE in tumor vs control mice by diet groups. p-value for differences between injection treatments in mice fed 2% kcal EPA+ DHA diet = 0.4597 (2-way

ANOVA) and p-value for differences between injection treatments in mice fed 0% kcal EPA + DHA diet = 0.9570 (2-way ANOVA)

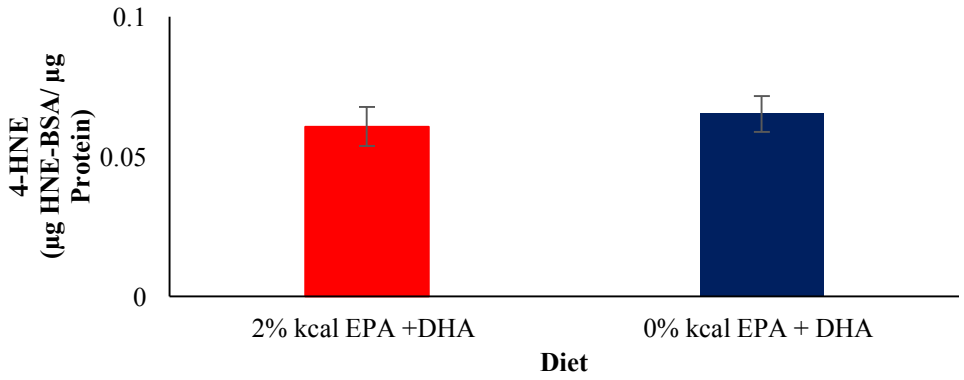


Figure 5: Comparison of liver 4-HNE by diet group without respect to injection treatment. p-value for differences between diet groups = 0.6345 (2-way ANOVA)

Chemotherapy mouse model

Mean (SEM) of 4-HNE values in the chemotherapy model mice are presented in Table 4. There were no statistically significant differences in mean 4-HNE by chemotherapy or control group (Figure 6). Using two-way ANOVA analysis, we explored trends for differences by diet groups (Figure 7), within diet and treatment groups (Figure 8).

Table 4: Descriptive analysis of liver 4-HNE in chemotherapy mouse model

Condition	**Variable**		**Mean of 4-HNE ± SEM (ug HNE-BSA/ugProtein)**
Comparison of all treatments, without respect to diet groups (Figure 6)	Vehicle		0.0876 ± 0.0114
	Chemotherapy		0.0817 ± 0.0114
Comparison of single diet to all treatments and comparison of	Low sucrose, 2% kcal EPA + DHA diet	Vehicle	0.1094 ± 0.0241
		Chemotherapy	0.0751 ± 0.0241
		Vehicle	0.0923 ± 0.0254

single treatment to all diets (Figure 7)	Low sucrose, 0% kcal EPA + DHA diet		
		Chemotherapy	0.0975 ± 0.0254
	High sucrose, 2% kcal EPA + DHA diet	Vehicle	0.0873 ± 0.0280
		Chemotherapy	0.0908 ± 0.0280
	High sucrose, 0% kcal EPA + DHA diet	Vehicle	0.0550 ± 0.0152
		Chemotherapy	0.0634 ± 0.1365
Comparison of all diets without respect to treatments (Figure 9)	Low sucrose	2% kcal EPA + DHA diet	0.0922 ± 0.0160
		0% kcal EPA + DHA diet	0.0949 ± 0.0160
	High sucrose	2% kcal EPA + DHA diet	0.0890 ± 0.0160
		0% kcal EPA + DHA diet	0.0596 ± 0.0169

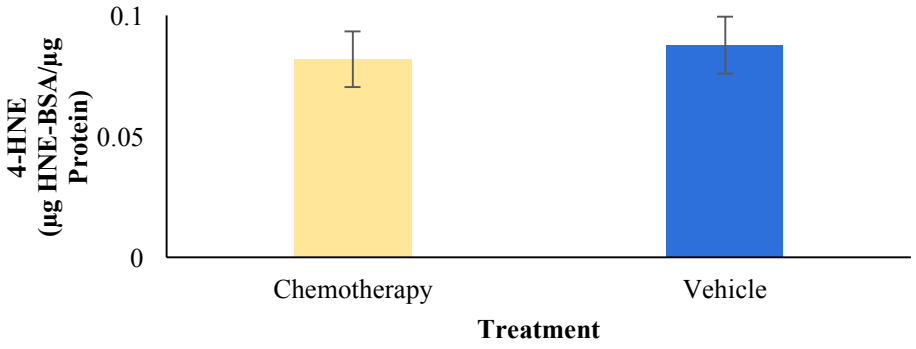
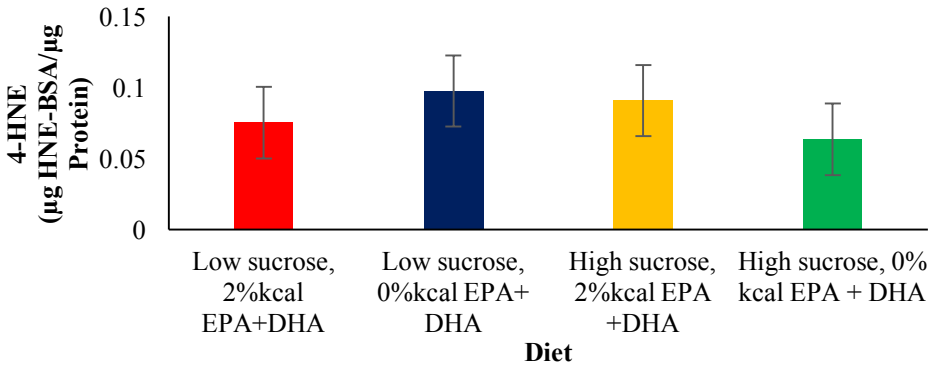


Figure 6: Comparison of liver 4-HNE in chemotherapy vs vehicle treated mice, without respect to diet group. p-value = 0.7199 (2 sample t-test)

a) Chemotherapy



b) Vehicle

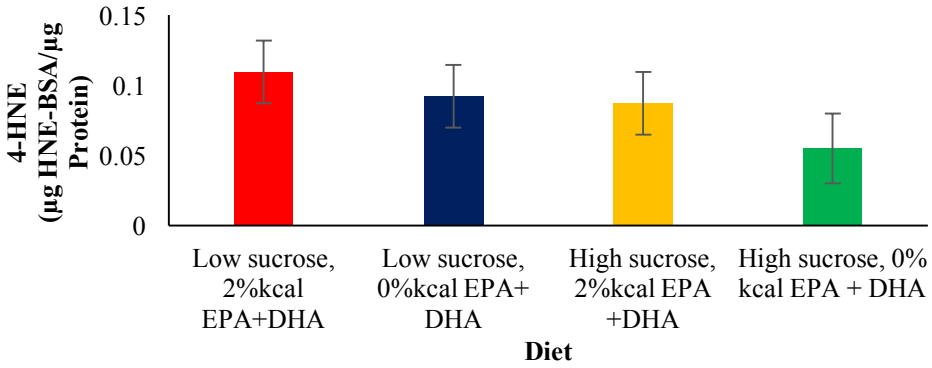


Figure 7a – Comparison of liver 4-HNE of all diet groups in chemotherapy treated mice. p-value for differences between all diets = 0.7698

Figure 7b – Comparison of liver 4-HNE of all diet groups in vehicle treated mice. p-value for differences between all diets = 0.4621

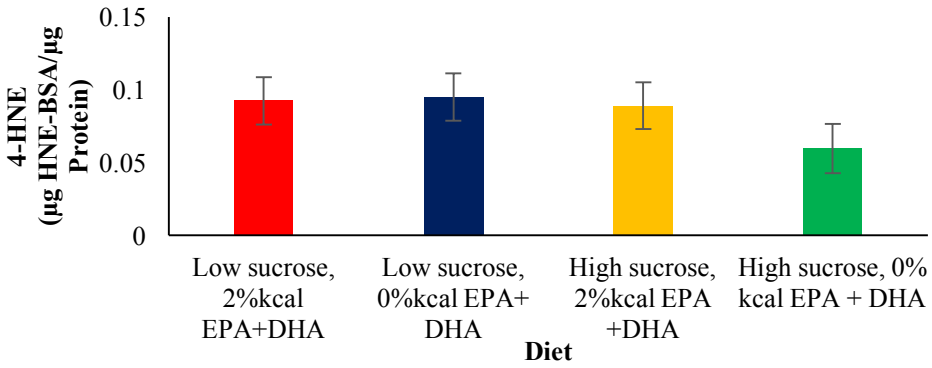


Figure 8 – Comparison of liver 4-HNE by diet group, without respect to treatment. p-value = 0.4228 (2-way ANOVA)

Discussion

The findings in this study suggested that there were no statistically significant differences between injection treatment groups or, diet groups, in liver 4-HNE levels when mice were fed varying levels of sucrose and omega-3 fatty acids and subjected to mammary tumor or chemotherapy injections.

Mammary tumor, omega-3 fatty acids and lipid peroxidation in the liver

The results from the mammary tumor model of this thesis differ from the studies conducted by Kikugawa et al., (2003), Gonzalez et al., (1993) and Olivo and Hilakivi-Clarke (2005). The studies showed that omega-3 fatty acids increased lipid peroxidation in mammary tumor in mice liver[4,5,11]. However, the thesis results showed no significant differences between omega-3 fatty acids and lipid peroxidation in mice liver.

Chemotherapy, omega-3 fatty acids and lipid peroxidation in the liver

The comparison of the results from the chemotherapy model of this thesis to the studies conducted by Bhattacharya et al., (2013) are different. The study found that omega-3 fatty acids were more susceptible to lipid peroxidation with the presence of chemotherapy[2] while this thesis found no differences in lipid peroxidation in liver.

Omega-3 fatty acids, sucrose and lipid peroxidation in the liver

The comparison of the results from the chemotherapy model of this thesis to the studies conducted by Ma et al., (2011) suggests different direction. The study suggested that sucrose might not have reduced omega-3 fatty acid's ability to prevent diet-induced accumulation of fat in the liver, which could decrease the amount of lipid peroxidation in the liver[8] while this thesis results were not significantly different.

The different results obtained in this thesis analysis compared to studies published might be because the mice were not exposed to as much chemotherapy treatment to produce the side-effects of treatment which includes lipid peroxidation. Moreover, there might be certain compounds or substances in the diets itself that were protecting the cells from lipid peroxidation. α-linolenic acid (ALA) content in the 0% EPA and DHA diet was much higher (7% of kcal) than the Adequate Intake of ALA for 19 to 70-year-old women (1.1grams/day or approximately 0.5% of an 1800 kcal/d diet), which could have helped protect the cells. The Adequate Intake levels are based on average consumption of ALA. 4-HNE is also a down-stream product of arachidonic acid (AA) instead of omega-3 fatty acids like ALA, EPA and DHA hence different biomarker of lipid peroxidation for this thesis could be assessed. The sample size of the two mice models were small as well and that could have given a relatively weaker power to detect significant relationships between the data of the study.

Conclusion

In conclusion, we found that various levels of sucrose and omega-3 fatty acid dietary modification did not cause lipid peroxidation in the liver to significantly differ in mouse models of breast cancer and chemotherapy. In regards to the efficacy of omega-3 fatty acid in limiting lipid peroxidation in both mammary tumor and chemotherapy models, further analysis of the models with different lipid peroxidation biomarkers should be done. A comparison between a low ALA diet with high EPA and DHA diets could give a clearer picture of differences in lipid peroxidation effects. In the future, statistical analysis using a three-way ANOVA looking at sucrose, omega-3 fatty acids and treatments should be done to identify if sucrose or omega-3 fatty acids alone compared to treatments would produce significant differences. A longer time frame of feeding could be investigated as well as numerous injection treatments, more closely mirroring chemotherapy in humans. The role and benefits of omega-3 fatty acids in helping to prevent oxidative stress in the liver is important to understand in order to help alleviate side-effects of patients who are going through chemotherapy. Hence, continuation of research involving efficacy of omega-3 fatty acids as well as interactions with different anticancer agents and sucrose should be studied.

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Appendix A

The protocol manual was prepared by Dr. Panchita Phuwamongkolwiwat-Chu

Protein Extraction

1. Label 2 sets of 2.5 mL Eppendorf tube each sample with different color markers (one set is used for homogenization and another one is for supernatant after centrifugation)
    a. Keep Eppendorf tubes on ice

2. Prepare 1X PBS from 10XPBS by estimating 1000 uL per sample (as needed)

3. Dissolve 1 tablet of protease inhibitor (Roche cat# 11836153001) into10 mL of 1xPBS
    a. Store on ice or in the fridge (once protease inhibitor added, the buffer is good for 1 week at 4°C)

4. Cut about 50 mg of brain tissue (Note: 5 -10 mg of liver tissue) on dry ice and record weight (keep frozen at all times)

5. Add the brain sample to the appropriate homogenization Eppendorf tube
6. Add 10x (v/w) 1XPBS with protease to labeled Eppendorf tubes on ice (Note: 100x v/w for liver)
    a. i.e. 50.6 mg (0.0506 g) of tissue = 506 uL of 1xPBS with protease

    Note: if the total volume is more than 1000 uL, first add 1000 uL for homogenization then add the rest volume after to prevent overflow during homogenization
7. Homogenize the sample using 5 mm homogenizer probe for 15-20 second at level 4

8. Immediately place Eppendorf tubes on ice and keep in ice till all samples finish

9. Wash the probe by running in plastic homogenization tubes containing 4 mL 1X PBS (1 set contains 4 tubes for washing after every 5 samples)

Repeat steps 4-9 for all samples

10. Once all samples are homogenized, incubate on the shaker in cold room for 1 hour

11. Centrifuge at 16100 rcf for 15 minutes at 4°C (cold room)

12. Use 200 or 1000 ul pipette to transfer supernatant (clear liquid on surface) to new Eppendorf tubes
    a. Must store on ice after transfer

13. Continue to BCA Protein assay or store at -80°C freezer for later

BCA Protein Assay (Pierce BCA Protein Assay Kit, Thermo Scientific Cat#23225)

(Note: If samples are from -80°C freezer, let samples thaw on ice)

1. Make Standards in microcentrifuge tubes (can be use again; store at RT)

Standard	**Concentration (ug/ml)**	**BSA (ul)**	**DI H2O (ul)**
0	0	0	300
1	125	18.75	281.25
2	250	37.5	262.5
3	500	75	225
4	750	112.5	187.5
5	1000	150	150
6	1500	225	75
7	2000	300	0

2. Set up 96 well plate as follows

	1	2	3	4	5	6	7	8	9	10	11	12
A	std 0	std 0	1	1	9	9	17	17	25	25	33	33
B	std 1	std 1	2	2	10	10	18	18	26	26	34	34
C	std 2	std 2	3	3	11	11	19	19	27	27	35	35
D	std 3	std 3	4	4	12	12	20	20	28	28	36	36
E	std 4	std 4	5	5	13	13	21	21	29	29	37	37
F	std 5	std 5	6	6	14	14	22	22	30	30	38	38
G	std 6	std 6	7	7	15	15	23	23	31	31	39	39
H	std 7	std 7	8	8	16	16	24	24	32	32	40	40

3. Make a working Reagent (~300ul per sample and standard)
    a. Mixing 50 parts of Reagent A with 1 parts of Reagent B (50:1 Reagent A / Reagent B, v/v)
    Example: 96 wells x 250 uL = 24,000 uL = 24mL
    Prepare 25 mL working solution:
    Reagent A = 25 mL: Reagent B = 0.5 mL (500 uL)

4. Add 25 uL of each standard/ to wells of 96 well plate (touch pipette tip to top of the wells)

**** For brain sample**: 5 uL of sample and 20 uL of 1 X PBS + Protease inhibitor buffer to each wells

5. Add 200 uL of Working Reagent to each well with multichannel pipette (touch pipette tip to left side of wells)

6. Place on shaker for about 10-15 seconds

7. Incubate @ 37°C for 30 minutes (incubator is at Room 362 D, please check with Dr. Julie C. before using)

8. Allow plate to cool to RT (~5 minutes) and check whether there is any bubble in the well or not. IF there is bubble, please use the clean pipet tip to blow air to remove the bubble.

9. Read absorbance at 562 nm (using BCA protocol in spectrometer program)

10. Continue to 4HNE ELISA or store samples in freezer in -80 for later

OxiSelect™ HNE Adduct Competitive ELISA Kit (Cat# STA-838)

DAY 1: Prepare HNE Conjugate Coated Plate:

1) Prepare 1X Conjugated Diluent from 100X Conjugated Diluent with 1X PBS (immediately before use)
2) Prepare 10 ug/ mL HNE Conjugate from 1.0 mg/ mL HNE Conjugate with 1X PBS (immediately before use)
3) Mix solution from step 1) and 2) to 1:1 ratio
4) Pipet 100 uL of the mixture of 1X Conjugated Diluent and 10 ug/ mL HNE Conjugate (step 3)) into each well
5) Cover the plate with Parafilm wrap and incubate overnight at 4°C in plate shaker (cold room)

DAY 2: Assay Protocol

Note:

- If samples are from -80°C freezer, let samples thaw on ice
- Remove HNE-BSA from -20 freezer and thaw on ice

1) Remove the HNE Conjugate Coated plate (from Day 1) from cold room
2) Discard the solution in the plate at wash sink
3) Wash with 200 uL 1X PBS, blot plate on the clean paper towel to remove excess fluid
4) Repeat step 2) -3) again

5) Pipet 200 uL of Assay diluent to each well and block for 1 hour on plate shaker in room temperature
6) While waiting, prepare 1X Wash buffer:
    a. Dilute 10X Wash buffer to 1X with Deionized water
    Example: 96 wells * 250 uL/well * 3 times * 2 steps = 144,000 uL = 144 mL
    Prepare 150 mL 1X Wash buffer

$C_1V_1 = C_2V_2$
10 (x) = 1(150)
X = 1(150)/10
= 15 mL of 10X Wash buffer in 135 mL Deionized water

    b. Swirl until homogeneity

7) Prepare label 2.5 mL Eppendorf tubes for HNE-BSA standards as below:

Standard	1 mg/mL HNE-BSA standard (uL)	Assay diluent (uL)	**HNE-BSA (ug/mL) Concentration**
1	80	320	200
2	200 of tube 1	200	100
3	200 of tube 2	200	50
4	200 of tube 3	200	25
5	200 of tube 4	200	12.5
6	200 of tube 5	200	6.25
7	200 of tube 6	200	3.13
8	200 of tube 7	200	1.56
9	0	200	0

    a. Vortex thoroughly
    b. Place on the ice at all time

8) Set of 96 well Plate as shown below

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	STD 1	STD 9	1	1	9	9	17	17	25	25	33	33
B	STD 2	STD 2	2	2	10	10	18	18	26	26	34	34
C	STD 3	STD 3	3	3	11	11	19	19	27	27	35	35
D	STD 4	STD 4	4	4	12	12	20	20	28	28	36	36

E	STD 5	STD 5	5	5	13	13	21	21	29	29	37	37
F	STD 6	STD 6	6	6	14	14	22	22	30	30	38	38
G	STD 7	STD 7	7	7	15	15	23	23	31	31	39	39
H	STD 8	STD 8	8	8	16	16	24	24	32	32	40	40

9) Pipet 50 uL of standards to 96 well (touch pipette tip to top of well)
10) Pipet 50 uL sample (touch pipette tip to top of well)
    Note: before pipet standards/samples vortex thoroughly before
11) Incubate for 10 minutes at room temperature on plate shaker
12) While waiting, prepare the diluted Anti-HNE antibody:
    - Dilute Anti-HNE antibody 1:1000

    Example: 96 wells * 50 uL/well = 4,800 uL ~ 5 mL
    Prepare 5 mL of Dilute Anti-HNE antibody

    C_1V_1 = C_2V_2
    1000 (x) = 1(5)
    X = 1(5)/1000
    = 0.005 mL of 1000 X Anti-HNE antibody

    So, 5 uL of **Anti-HNE** to 4.995 mL of **Assay Diluent**

13) After incubation, pipet 50 uL of the diluted Anti-HNE antibody (step 12) to each well.
14) Incubate for 1 hour at room temperature on plate shaker
15) Once plate is through incubating, wash wells 3 times with 250 uL of **1X Wash Buffer** using multichannel pipette

16) While waiting, prepare the diluted Anti-HNE antibody:
    - Dilute Secondary Antibody 1:1000

    Example: 96 wells * 10 uL/well = 9,600 uL ~ 10 mL
    Prepare 10 mL of Dilute Anti-HNE antibody

    C_1V_1 = C_2V_2
    1000 (x) = 1(10)
    X = 1(10)/1000
    = 0.01 mL of 1000 X Anti-HNE antibody

    So, 10 uL of **Anti-HNE** to 9.990 mL of **Assay Diluent**

17) Pipet 100 uL of the diluted Secondary Antibody (step 16) in each well (touch pipette tip to right side of well)
18) Incubate for 1 hours at RT on orbital shaker
    (Note: While incubating; warm Substrate solution to room temperature)
19) Repeat washing step as step 15 above.
20) Pipet 100 uL of Substrate Solution to each well
21) Incubate for 20 minutes at room temperature on plate shaker
    Note: *watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation* (I did 20 minutes)
    Note: turn on the plate reader and set the temperature at 37 °C
22) Add 100 uL of **Stop Solution** to each well (tough pipette tip to right side of well)
23) Read absorbance at 450 nm immediately

Appendix B

Mouse Diet Formulations were prepared by Research Diets, Inc.

	0% EPA+DHA/ LOW SUCROSE	0% EPA+DHA/ HIGH SUCROSE	2% EPA+DHA/ LOW SUCROSE	2% EPA+DHA/ HIGH SUCROSE
Ingredient	gm/kg	gm/kg	gm/kg	gm/kg
Casein	232.5	232.5	205	205
DL-Methionine	3	3	3	3
Corn Starch	450	100	450	100
Sucrose	100	500	100	500
Maltodextrin10	100	50	100	50
Cellulose	50	50	50	50
Soybean Oil	90	90	41	41
MEG-3, 30% Powder	0	0	81	81
Mineral mix	35	35	35	35
Vitamin mix	10	10	10	10
Choline	2	2	2	2
Protein (gm%)	19	19	19	19
Carbohydrate (gm%)	61	61	61	61
Fat (gm%)	8	8	8	8
Protein (kcal%)	19	19	19	19
Carbohydrate (kcal%)	61	61	61	61
Fat (kcal%)	19	19	19	19

Appendix C

Fatty acid composition of mouse diets prepared by Kate Ormiston from Dr. Orchard's lab

	Diets							
Dietary Fatty Acid	**NO EPA+DHA/ LOW SUCROSE**		**NO EPA+DHA/ HIGH SUCROSE**		**2% EPA+DHA/ LOW SUCROSE**		**2% EPA+DHA/ HIGH SUCROSE**	
	Mean %	***SD***	***Mean %***	***SD***	***Mean %***	***SD***	***Mean %***	***SD***
C14:0	0.3127	0.0056	0.3465	0.0183	4.0342	0.0655	4.0396	0.1155
C16:0	10.8571	0.0044	10.8156	0.0162	15.6643	0.0515	15.6578	0.1455
C16:1n7	0.1246	0.0102	0.1256	0.0121	4.7284	0.0682	4.7533	0.1353
C16:2n4	ND		ND		0.6467	0.0136	0.6746	0.0129
C16:3n4	0.0585	0.0033	0.0580	0.0018	0.7979	0.0132	0.7923	0.0207
C18:0	3.7595	0.0075	3.7911	0.0128	3.7329	0.0262	3.8003	0.0131
C18:1n9	19.9944	0.0661	19.9874	0.0771	14.3479	0.0526	14.5019	0.1299
C18:1n7	1.4386	0.0437	1.4651	0.0507	2.1407	0.0437	2.1796	0.0354
C18:2n6	55.7595	0.0116	55.7239	0.0613	28.0789	0.4165	27.4273	0.8709
C18:3n6	ND		ND		0.1622	0.0088	0.1744	0.0152
C18:3n3	7.1302	0.0301	7.1640	0.0585	4.0564	0.0457	3.9617	0.0978
C18:4n3	ND		ND		2.1081	0.0256	2.1021	0.0458
C20:0	0.2380	0.0153	0.2492	0.0068	0.4335	0.0129	0.4489	0.0220
C20:1n9	0.1587	0.0057	0.1676	0.0039	0.8631	0.0403	0.9112	0.0393
C20:2n6	0.0424	0.0044	ND		0.1139	0.0040	0.1141	0.0067
C20:3n6	ND		ND		0.0954	0.0057	0.0990	0.0071
C20:4n6	ND		ND		0.4834	0.0083	0.4938	0.0109
C20:4n3	ND		ND		0.5428	0.0136	0.5548	0.0225
C20:5n3	ND		ND		9.2107	0.1225	9.3503	0.2936
C22:4n6	0.0989	0.0152	0.0988	0.0067	0.1218	0.0623	0.1128	0.0966
C22:5n6	ND		ND		0.1417	0.0131	0.1481	0.0094
C22:5n3	0.0740	0.0256	0.0602	0.0035	0.8908	0.0689	0.9321	0.1051
C22:6n3	ND		ND		6.6043	0.1001	6.7700	0.2156
Total n-3	7.2042	0.0501	7.2041	0.0238	23.4131	0.3142	23.6710	0.5725
Total n-6	55.8537	0.0765	55.7897	0.0145	29.1974	0.4603	28.5695	0.9279
n-3/n-6	0.1290	0.0011	0.1291	0.0004	0.8021	0.0231	0.8296	0.0470
n-6/n-3	7.7533	0.0642	7.7442	0.0254	1.2474	0.0364	1.2080	0.0685